

THE GROWTH OF MICRO-ORGANISMS *IN VIVO* WITH
PARTICULAR REFERENCE TO THE RELATION
BETWEEN DOSE AND LATENT PERIOD

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(With 12 Figures in the Text)

INTRODUCTION

The multiplication of micro-organisms *in vivo* can be studied quantitatively by performing viable counts on infected animals, or by determining the relation between dose and latent period (the interval between inoculation and response). We have applied both methods to mice given *Salmonella typhimurium* by intra-peritoneal injection. The interpretation of the results for hosts responding to inoculation depends on the proportion of administered organisms whose multiplication is believed to be the cause of the response. This point has been discussed by Meynell & Stocker (1957), who suggested that the proportion could be predicted from a general hypothesis (the hypothesis of independent action) which relates several other features of infective systems. These are, the increasing probability of a response with increase in dosage, and the isolation of pure clones of organisms from hosts inoculated with limiting dilutions (Kunkel, 1934; Meynell & Stocker, 1957; Meynell, 1957*a*); the shape of the dose-response curve and the relatively high variability in response observed in infectivity titrations using an all-or-none response (Meynell, 1957*b*); the effect on response of subdivision of the total number of organisms inoculated (Goldberg, Watkins, Dolmatz & Schlamm, 1954); and the approximate constancy of the latent period reported below for doses equal to or less than the ED₅₀. The latter provides a new test of the hypothesis.

The results of previous experiments in which mice were inoculated with mixtures of distinguishable variants of a given salmonella (Meynell & Stocker, 1957) suggested that death following inoculation of doses ≥ 1 LD₅₀ of a strain of intermediate virulence was ultimately due to the multiplication of a fraction of the inoculum far larger than that predicted from the size of the ED₅₀ by the hypothesis of independent action. The discrepancy was attributed to the effect of a breakdown in resistance caused by the predicted fraction which enabled other inoculated organisms or their progeny to multiply progressively and to contribute to the number

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of organisms causing death. These experiments, although disturbed to some extent by the breakdown in resistance, were not nullified, since the results were in general agreement with expectation. However, when the results of the present experiments were examined, it became clear that they were not explicable on the simple form of the hypothesis, which makes no allowance for a breakdown, but could be easily accounted for if the hypothesis was extended to include this effect. A short account of the hypothesis of independent action in its simplest and extended forms is therefore given, followed by a model for the multiplication of organisms *in vivo* suggested by the relation between dose and latent period observed in infective systems.

The hypothesis of independent action

The hypothesis first makes the assumption that the fates of individual inoculated organisms are randomly determined: for example, that the death or survival of a given inoculated organism and its progeny is decided by chance events occurring *in vivo*. It follows that each inoculated organism and its progeny have a chance of experiencing an event or a succession of events *in vivo* which will permit sufficient multiplication to initiate a response such as death or a lesion of specified size. This chance, denoted by p ($1 \geq p > 0$) can be estimated experimentally by determining the relation between dose and the occurrence of a quantal response (Meynell, 1957*b*). If the organisms are of maximum virulence, the inoculation of any single organism will invariably lead to a response so that $p = 1$. But, if the organism is of intermediate virulence, more than one organism will usually have to be inoculated before a response follows and the value of p is therefore less than unity. Hence, if a host is inoculated with d organisms of either maximum ($p = 1$) or intermediate virulence ($1 > p > 0$), the mean number of organisms which will multiply sufficiently to initiate a response will be pd . Such organisms will be referred to as 'effective' without implying that they necessarily differ in either phenotype or genotype from other inoculated organisms.

It is next necessary to consider how a response might be initiated by effective organisms. The simple form of the hypothesis postulates that, whatever the virulence of the organisms ($1 \geq p > 0$), the response is due solely to the multiplication of the postulated effective fraction of the inoculum, p . This must be true for organisms of maximum virulence where $p = 1$ and every inoculated organism is effective; and will also be true of partially resistant hosts which invariably respond to any organism which succeeds in passing a surface tissue, such as the respiratory or intestinal epithelium (Meynell, 1957*a*). But, as mentioned above, earlier experiments indicate that the simple form of the hypothesis does not apply to attenuated salmonella ($p < 1$) deposited within the host tissues, when it is likely that the initially effective organisms sooner or later produce a fall in resistance which enables initially ineffective organisms to multiply and to contribute to the total number of organisms whose multiplication eventually produces the response. Hence, in the latter infections, f , the fraction of the inoculum whose multiplication determines the length of the latent period will be larger than the effective fraction, p , predicted by the hypothesis in its simple form. In fact, the results of the present experiments suggest that f could comprise the entire inoculum.

Further assumptions are that inoculated organisms are completely independent, and that p is constant for a given system and is unaffected by the number of organisms inoculated. This assumption is plausible in the early stages of an infection, as in most systems nontoxic doses of organisms are distributed over a relatively large area. It is unlikely, for example, when blood-born organisms are phagocytosed by cells of the reticulo-endothelial system, that any single cell will contain more than one organism. Furthermore, the number of organisms inoculated is usually far less than either the toxic LD_{50} of killed organisms (*ca.* 10^{10} for gram-negative bacilli) or the smallest number of killed organisms (*ca.* 10^7) which can be shown to affect the LD_{50} of attenuated living organisms of the same species inoculated at the same time (Maaløe, 1948; Rowley, 1954). Although alternative, and perhaps more realistic, hypotheses could be stated on the assumption that p varies with the size of the dose, they have the disadvantage of not yielding precise predictions, as the relation of p to dose is unknown. It is therefore assumed here provisionally that the organisms are completely independent and that p is constant for all doses.

A model for the production of a response by the multiplication of micro-organisms

A search of the literature, summarized in Table 1, showed that for those hosts in which a response occurred, average latent period was linearly related to logarithm of dose whatever average was measured, e.g. the arithmetic mean, the geometric mean or the median. Reports in which the reciprocal of the harmonic mean* has been used which varies inversely with the actual latent period, have not been included but the data from three of these (Gard, 1940; Ipsen, 1941; Mandel & Racker, 1953) were recalculated and gave a linear relation between log dose and latent period.

The linear relation observed between log dose and latent period suggested that a model proposed by several authors (Bonezzi, Cavalli & Magni, 1943; Gard, 1943; Youmans & Youmans, 1951) for the multiplication of effective organisms in particular systems was in fact, generally applicable. This model may be applicable to any system in which a response is produced by the multiplication of a constant proportion, f , (≤ 1) of inoculated organisms, whether or not the proportion is that predicted by the simple form of the hypothesis of independent action.

The simplest form of the model postulates (a) that organisms increase *in vivo* at a constant rate so that their number rises exponentially, and (b) that the response occurs when the total number of organisms reaches or exceeds a critical figure, C . Hence

$$N_t = fde^{it}, \quad (1)$$

where N_t is the number of organisms descended from the fraction f at time t after inoculation, d is the mean total number of organisms inoculated, f is the mean proportion of inoculated organisms whose growth determines the length of the

* The reciprocal of the harmonic mean, introduced by Gard (1940), has the advantage for an assay that its variance is constant and independent of the mean, and that its value can take into account those hosts which do not respond.

latent period, i is their mean rate of increase *in vivo*; and e is the base of natural logarithms. (1) gives

$$t = \{\log_e N_t - \log_e (fd)\} / i \quad (2)$$

which, if C exists, gives a straight line when log dose is plotted against latent period. The model is illustrated in Fig. 1*a*, which shows the postulated manner of growth *in vivo*, and in Fig. 1*b*, which shows the usually realized form of predicted relation between log dose and latent period.

Table 1. *Infective systems in which average* latent period is linearly related to logarithm of dose*

Infective agent	Host	Response	Source
Papilloma virus	Rabbit	Formation of local lesion	Bryan & Beard (1939, 1940)
Bronchopneumonia virus	Mouse	Death	Gonnert (1942)
<i>B. anthracis</i>	Mouse	Death	Bonezzi, Cavalli & Magni (1943)
<i>Str. pneumoniae</i>	Mouse	Death	Cavalli & Magni (1943)
Psittacosis virus and other viruses	Chick embryo	Death	Golub (1948)
<i>Myco. tuberculosis</i>	Mouse	Death	McKee, Rake, Donovick & Jambor (1949)
Feline pneumonitis virus and other viruses	Chick embryo	Death	Weiss (1950 <i>a, b</i>)
<i>Coxiella burnetii</i>	Chick embryo	Death	Ormsbee, Lackman & Pickens (1951)
<i>Trep. pallidum</i>	Rabbit	Presence of organism in skin lesion	Magnuson, Rosenau & Greenberg (1951)
<i>Myco. tuberculosis</i>	Mouse	Death	Youmans & Youmans (1951)
<i>Myco. tuberculosis</i>	Mouse	Death	Stewart & Tamargo-Sanchez (1952)
Murine pneumonitis virus	Mouse	Death	Gogolak (1953)
Meningo-pneumonitis virus	Mouse	Death	Crocker (1954), Crocker & Bennett (1955)
<i>Br. tularensis</i>	Various	Death	Bell, Owen & Larson (1955)
Rift Valley Fever virus	Mouse	Death	Mims (1956)
<i>L. icterohaemorrhagiae</i>	Guinea-pig	Death	Faine (1957)

* The Table only includes systems where the latent period is given as an average which increases directly with prolongation of the latent period, such as the arithmetic mean or the geometric mean.

It will be noted that

- (a) the mean latent period for hosts responding to doses $\leq 1 \text{ ED}_{50}$ tends almost always to become constant as each of these responses is assumed to be initiated usually by the multiplication of only one effective organism if the hypothesis of independent action is valid (v.i.);
- (b) according to the model, $t = 0$ when the dose $\geq C$ (see dashed line in Fig. 1*b*). This is clearly implausible, since no dose is likely to cause an instantaneous response. The line is therefore expected to asymptote to the dose axis as shown by the continuous line in Fig. 1*b*, and has been found to do so by

Gonnert (1942), Bonezzi *et al.* (1943), Cavalli & Magni (1943), Mandel & Racker (1953), and by Smith & Westgarth (1957). In virus infections, the same effect could follow saturation of the host receptors available for infection at the time of challenge (Gogolak, 1953);

- (c) (i) the rate of increase, i , is assumed constant, i.e. independent of the size of the dose. Changes in i following inoculation may be detected in the distribution of the latent periods of members of a given dose group (see Discussion), (ii) the rate of increase need not equal the rate of multiplication *in vivo*, since it would be constant if organisms both multiplied and were inactivated at constant rates; (iii) the value of i for those organisms whose growth determines the length of the latent period (i.e. for the fraction, f) can be estimated from the slope of that part of the latent period/log dose curve which is linear for doses $\geq 1 \text{ ED}_{50}$ as $i = (\log_e d_2 - \log_e d_1)/(t_1 - t_2)$ from (2). Hence, i can be estimated without knowledge of either p , f , or C ;
- (d) neither a lag before multiplication begins, nor a lag between the time C is reached and the occurrence of the response, affect the validity of the model provided both are constant.

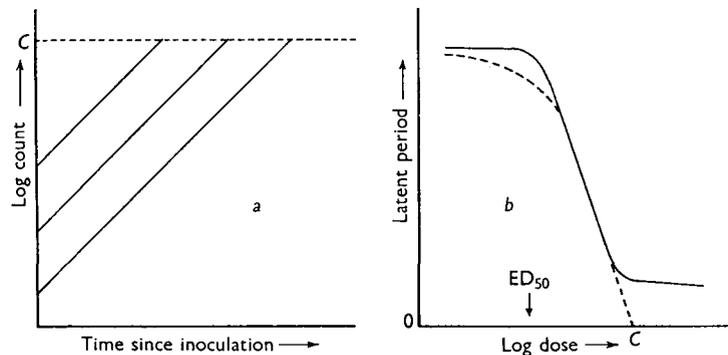


Fig. 1. The model postulates that effective organisms increase *in vivo* exponentially and that the response will occur when their number reaches a constant figure. The growth curves would have the form shown in Fig. 1a, yielding, for doses $\geq 1 \text{ ED}_{50}$, the linear relation between latent period and log dose shown in Fig. 1b. The slope of this part of the curve is not fixed and is assumed to equal the reciprocal of the rate of increase *in vivo*. The constancy of the latent period for doses $\leq 1 \text{ ED}_{50}$, and for very large doses, is discussed in the Introduction.

The above model applies to any type of quantal response produced by the presence of a critical number of self-reproducing cells: such as death from leukaemia (MacDowell, 1936) or tumour growth (Prince, Littell & Ginsberg, 1957); the formation of a localized tumour (Reinhard, Goltz & Warner, 1945); the production of a chosen degree of pneumonia (Horsfall & Ginsberg, 1951); or, possibly, to the production of a lethal amount of exotoxin by a localized collection of bacteria. Gledhill (1956) has proposed an extension of this model for the effect of superinfection with *Eperythrozooa* on the latent periods of mice inoculated with mouse hepatitis virus.

A new test of the hypothesis of independent action based on the predicted relation between dose and latent period for doses $\leq 1 \text{ ED}_{50}$

The simple form of the hypothesis of independent action predicts that the mean number of effective organisms per host is pd , where p is the mean probability per inoculated organism of being effective and d is the mean number of organisms inoculated. When pd is small (say, ≤ 0.5), the number of effective organisms for many hosts will be 0, so that these hosts will not respond, whereas for others it will be 1, or less often, 2, 3, If the hosts do not differ in resistance, $pd \sim 0.7$ for a dose of 1 ED_{50} and it can be predicted from the Poisson series that *ca.* 70% of the responses will each be initiated by the multiplication of only one effective organism (Meynell & Stocker, 1957, Fig. 2). This percentage will increase as dosage is reduced further. Hence, most hosts responding to doses $\leq 1 \text{ ED}_{50}$ should respond after much the same length of time, since each will usually be responding to the multiplication of only one effective organism.

The predictions are more complex if the hosts differ in resistance because the proportion of organisms effective will then not be calculable from the Poisson series; and also because the present experiments show that the rate of increase *in vivo* of the effective organisms appears to be inversely related to the size of the ED_{50} . In general, it seems that for doses $\leq 1 \text{ ED}_{50}$ the log dose/latent period curve for heterogeneous hosts will be displaced downwards relative to the curve for homogeneous hosts of the same mean resistance (as shown by the left-hand dotted line in Fig. 1*b*).

Viable counts were also used to test the assumptions of the model. This course may be misleading for, if killing occurred *in vivo* at a relatively high rate, the number of dead organisms might be considerable and might contribute significantly to the critical number of organisms, C . However, the growth curves obtained by viable counts in this and other systems appear to have the form postulated by the model so that many infective systems behave as if only viable organisms determined the length of the latent period.

MATERIALS AND METHODS

Organisms. The following strains of *Salm. typhimurium* were used: SL 216 and SL 219, which are equal in virulence and are descended from strain SW 351 (Meynell & Stocker, 1957); strain 533 obtained from Dr G. Furness; and the streptomycin sensitive strain GLeB, (GLeBStr-), (Meynell, 1957*a*). *Salm. paratyphi* B, strain SL 162, carrying flagellar antigen *b* (Meynell & Stocker, 1957) was also used. All these strains are prototrophic. Challenge doses in either the logarithmic or the stationary phase of growth were prepared from dried stock cultures as described by Meynell & Stocker (1957). Dilutions of the cultures were kept in an ice-bath to prevent multiplication while the mice were being inoculated.

Mice. Female mice, weighing 18–25 g., of three lines were used: albino mice of the same line bred either at the Postgraduate Medical School or by F. H. Evans, 54*a* Stockwell Green, London, S.W. 9; Webster BSVS mice bred at the London

School of Hygiene and Tropical Medicine; and albino mice bred by A. Tuck and Son, The Mousery, Rayleigh, Essex. All these strains were free from disease except for the mice bred at Hammersmith, which at the time of Expts. 7 and 8 were suffering from chronic ectromelia. No deaths occurred amongst uninoculated mice in these experiments and no aberrant deaths were observed in the test groups. Only one mouse showed cutaneous lesions during the experiments, which ran concurrently, and this was discarded. All mice were fed on Diet 41 and were usually kept on sawdust in groups of three to seven mice in glass bowls of diameter 4 or 9 in., which were changed at least once a week.

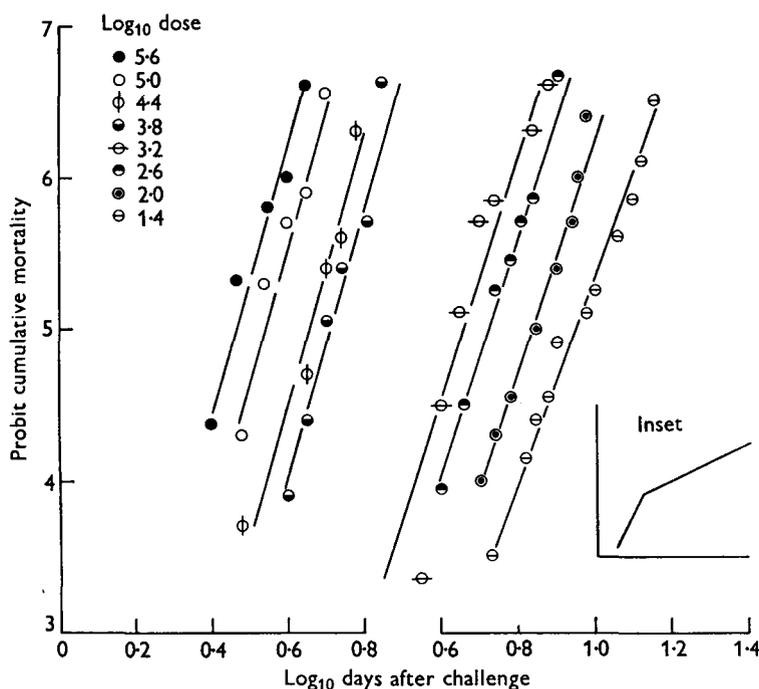


Fig. 2. Observed distribution of death times for the members of eight dose-groups in Expt. 5. Probit cumulative mortality has been plotted against logarithm of time since inoculation. The observed linear relation shows that the logarithms of death times in each dose group are normally distributed.

Inset. The relation observed in some other infections (see Discussion) when the observations are plotted in the same way. The discontinuity is attributed to a fall in the rate of increase of the organisms *in vivo* at the corresponding time after inoculation.

Recording of deaths and calculation of \bar{T} , the geometric mean death time. The pots were searched every 12 hr. in Expts. 1-6 and daily in Expts. 7 and 8.

As the logarithms of individual death times were found to be normally distributed (Fig. 2), the geometric mean death time, \bar{T} , has been calculated for each dose group. $\bar{T} = \text{antilog} (\log t_1 + \log t_2 + \dots + \log t_n)/n$, where t_n is the death time of the n th mouse. Each death was supposed to have occurred halfway between the time the mouse was found dead and the time of the previous search.

Viable counts. Mice were killed with chloroform and samples collected using

a sterile technique. The heart, lungs, spleen, liver, kidneys and peritoneal washings were transferred to a grinding tube made from precision-bore glass tubing, the total volume of the sample being made up to 10 ml. with quarter-strength Ringer's solution. The organs were then disintegrated by insertion of a Teflon grinder (Pierce, Dubos & Schaefer, 1953) revolving at 5300 r.p.m., this stage being carried out in a safety cabinet fitted with an extraction fan and an ultra-violet sterilizing lamp. No intact host cells could be seen in films of suspensions treated in this way. The suspension was finally diluted and samples of 0.2 ml. from successive tenfold dilutions were spread on dried plates of Lemco agar. Colonies were counted after 18–24 hr. incubation at 37° C. The counts are expressed as the total number of viable organisms recovered from each mouse.

RESULTS

Observed relationship between dose and death time

Eight experiments were done with mice given *Salmonella typhimurium* by intraperitoneal injection. The strains of mice and of organisms used are summarized in Table 2 which also gives the value of the LD₅₀, and the value of *i* and the presumed doubling time *in vivo* of the fraction, *f*, of inoculated organisms estimated from the slope of the death time/log dose curves. The data for Expts. 4–8 have been deposited in the Science Museum Library, South Kensington, London, S.W. 7.

Table 2. *Summary of experiments in which mice were given Salmonella typhimurium by intraperitoneal injection*

Expt. no.	Strain of mouse	Strain of <i>Salm. typhimurium</i>	LD ₅₀	<i>i</i> (hr.)	Doubling time (hr.)
1	PGMS	SL 216 + SL 219	320	0.045	15.5
2, 3	Tuck	533	3.2 × 10 ⁶	0.019	36.5
4	BSVS	SL 216 + SL 219	3	0.214	3.2
5	BSVS	SL 216	3	0.13	5.3
6	PGMS	SL 216 + SL 219	3	0.09	7.7
7	PGMS	GLeB	40	0.72	0.9
8	PGMS (vaccinated)	GLeB	3 × 10 ³	0.08	8.7

The values for *i*, the mean rate of increase *in vivo*, and for the doubling times were obtained from the slopes of the death time/log dose curves (Figs. 3–7) by the method described in the Introduction. The shortest doubling time observed *in vitro* is 0.5 hr.

The relationship for doses equal to or less than the LD₅₀. There are several difficulties in obtaining relatively precise estimates of \bar{T} for doses ≤ 1 LD₅₀. First, only a small proportion of hosts respond so that to obtain even a small number of responses, a large number of hosts must be challenged by each dose. The members of each dose group have therefore to be kept in groups with

the risk of cross-infection which was known to occur in Expt. 1, where uninoculated and inoculated mice were kept together in the same pots. Secondly, individual arithmetic latent periods become more scattered as dosage is reduced and the estimate of \bar{T} becomes less precise. Thirdly, it has to be assumed that resistance does not fluctuate during an experiment lasting up to 7 weeks. For example, a fall in resistance is suspected from $T = 72$ onwards in Expt. 3, chiefly amongst the mice given 10^5 organisms. Both mice dying on $T = 72$ came from a pot in which no deaths had previously occurred and the three succeeding deaths ($T = 74, 75$ and 76) occurred in the same pot. As a public holiday covered the period $T = 70-79$, these deaths were probably accidental and the experiment is assumed to have ended at $T = 70$. Table 5 shows that only one death in the other dose groups is excluded from the calculations.

In Expt. 1 (Table 3, Fig. 3) the LD_{50} was 320 organisms, estimated by Thompson's (1947) method and in Fig. 3 the values of \bar{T} clearly tend to become constant for doses \leq the LD_{50} . When the experiment was repeated about 3 months later

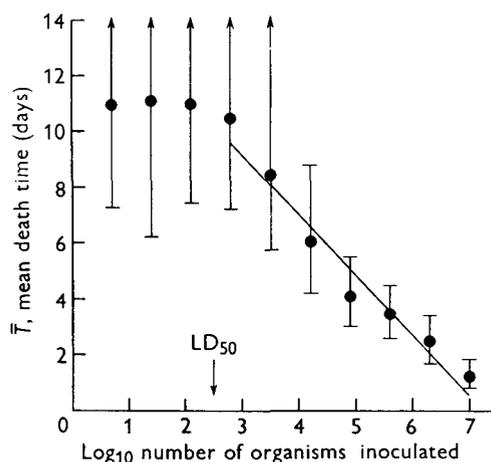


Fig. 3. Expt. 1. Geometric mean death time, \bar{T} , plotted against logarithm of the number of viable organisms inoculated. The vertical lines passing through each point in this and the succeeding Figs. show the range $\bar{T} \pm \text{antilog } 1.95 \text{ s.e. } (\log_{10} \bar{T})$. \bar{T} tends to constancy at doses $\leq 1 LD_{50}$ ($10^{2.5}$ organisms), as predicted. A straight line has been fitted by eye to the points for doses $\geq 1 LD_{50}$.

(Expt. 6), the LD_{50} had fallen to 3 organisms, presumably owing to a spontaneous change in resistance as observed with *Salm. typhi* by Bacon, Burrows & Yates (1951). As the LD_{50} did not increase in later titrations, Expts. 2 and 3 were performed with other mice and another strain of *Salm. typhimurium*.

Expts. 2 and 3 (Tables 4, 5) are plotted together in Fig. 4, since the dose-mortality and death time/log dose curves were similar. The LD_{50} was 3.2×10^6 organisms (estimated by eye from a plot of probit mortality against log dose). Both experiments gave similar values of \bar{T} for doses $\geq 1 LD_{50}$ and, in Expt. 2, \bar{T} appeared to become approximately constant for doses $\leq 1 LD_{50}$, as predicted. In Expt. 3 the two values for doses $\leq 1 LD_{50}$ are both greater than the value for $1 LD_{50}$ but are approximately constant.

The relationship for doses $\geq 1 \text{ LD}_{50}$. Figs. 5-7 show the results of Expts. 4-6. Expts. 1-6 probably fulfill the predictions of the model although the fit of the points to a straight line is not good. In every experiment, it would be difficult to draw a straight line through the confidence limits for the values of \bar{T} ; so that log

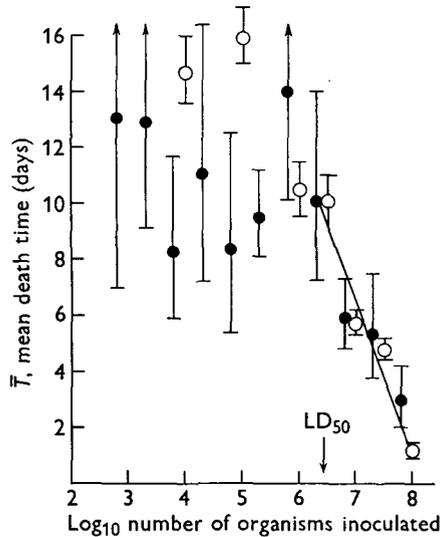


Fig. 4. \bar{T} plotted against logarithm of dose for Expts. 2 (solid points) and 3 (open points), showing that \bar{T} tends to constancy for doses $\leq 1 \text{ LD}_{50}$ ($10^{6.5}$ organisms).

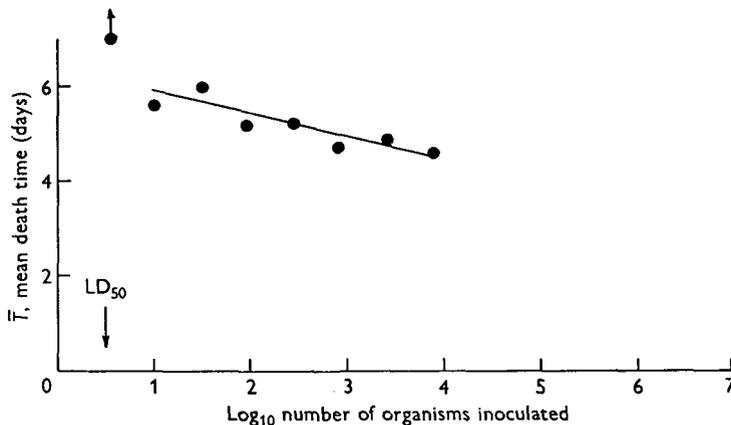


Fig. 5. Expt. 4. \bar{T} plotted against log dose.

dose would be unlikely to be linearly related to \bar{T} , as postulated, if the mice were identical in resistance and sampling error alone was responsible for the deviation of the points from linearity. In fact, the mice presumably differed in resistance in every experiment so that \bar{T} and log dose may well have been linearly related, this being more likely than appears at first sight from the Figures. As the curves did not show a systematic departure from linearity, straight lines have been fitted to the points by eye in order to estimate the slope and the value of i .

Expts. 5 and 6 (Figs. 6, 7) gave curves with an inflexion at a dose of 10^3 organisms. We cannot provide a plausible explanation for this and as it is not present in any of the other curves or in those reported in the literature, it may well be due to coincidence.

The first six experiments suggested that the greater the resistance of the mice, the more slowly did the organisms increase *in vivo*, since the slope of the death time/log dose curve was directly related to the size of the LD_{50} . It was particularly interesting to see that the spontaneous fall in resistance of the PGMS mice between Expts. 1 ($LD_{50} = 320$ organisms) and Expt. 6 ($LD_{50} = 3$ organisms) was accompanied by a decrease in slope. The LD_{50} and death time/log dose curve were therefore determined for a group of mice, half having been actively immunized with a heat-killed vaccine, which was challenged by doses drawn from the same set of dilutions (Expts. 7, 8). Vaccination increased the LD_{50} about 100 fold (Table 2) and also increased the slope of the death time/log dose (Fig. 8) as expected. Both curves were approximately linear.

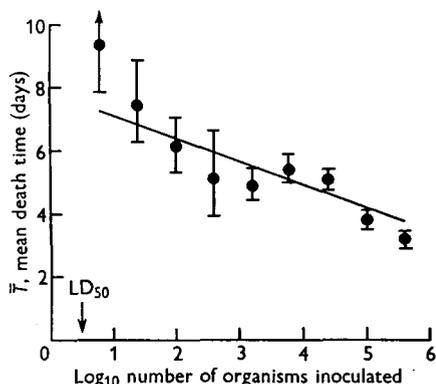


Fig. 6. Expt. 5. \bar{T} plotted against log dose.

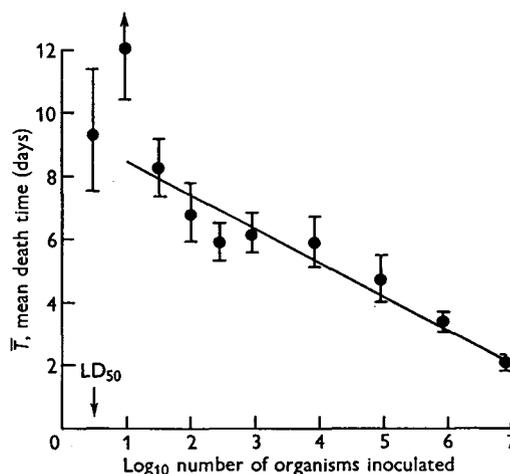


Fig. 7. Expt. 6. \bar{T} plotted against log dose.

Viable counts on infected mice

These counts were done with two objects. First, to see if the terminal viable count was constant and independent of dose; and secondly, to see if the multiplication of the effective organisms could be distinguished and, if so, if it occurred in the manner postulated by the model.

Counts performed immediately after death. Sick mice were watched and a viable count done as soon as possible after breathing had stopped, usually within 30 min. The counts were made on PGMS mice from Expt. 1 ($LD_{50} = 320$ organisms) and on BSVS mice from Expt. 4 ($LD_{50} = 3$ organisms). Fig. 9 shows that the terminal viable count was approximately constant at $10^{8.75}$ organisms in both experiments, regardless of dosage and the resistance of the mice, which strongly suggested that this figure is the critical concentration postulated by the model.

Counts performed during the course of infection. If the organisms are of low

virulence ($p < 1$) and act independently, only a small fraction (p on the average) of the total number inoculated is assumed to be effective. Hence, if the growth of this small fraction is to be distinguished from that of the much larger fraction of 'ineffective' organisms by means of viable counts, the ineffective organisms must at least not increase after inoculation.

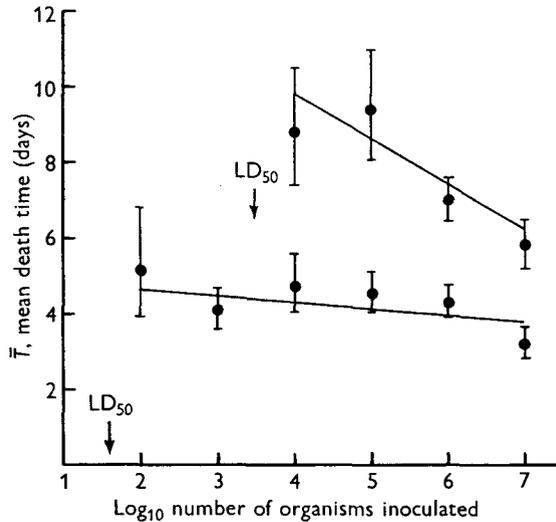


Fig. 8. Expts. 7 (lower curve) and 8 (upper curve) showing \bar{T} plotted against log dose for normal and vaccinated mice, respectively, inoculated with the same strain of *Salmonella typhimurium*.

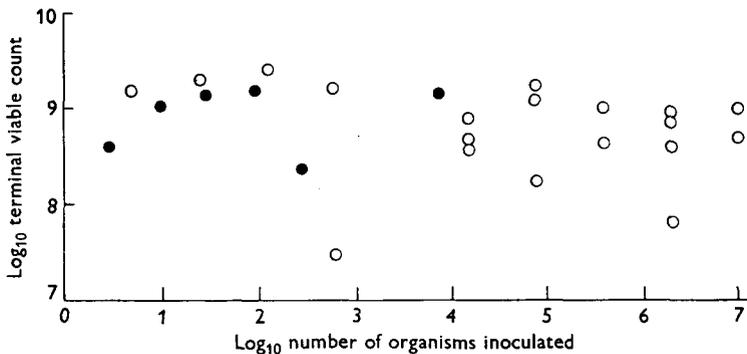


Fig. 9. Logarithm of observed terminal viable count plotted against logarithm of dose, showing that the former is approximately constant with mean of $10^{8.75}$, for all doses given to two lines of mice of differing resistance. Counts from Expt. 1 ($LD_{50} = 320$ organisms; open points) and Expt. 4 ($LD_{50} = 3$ organisms; solid points).

The first counts were done on mice inoculated in Expt. 1 with the result shown in Fig. 10a-c. The LD_{50} in the main experiment was 320 organisms so that p was *ca.* 2×10^{-3} . None of the curves are consistent with the sole multiplication of the postulated effective fraction. The curve for mice given $10^{3.5}$ organisms differs from the other curves as the count began to fall after the third day and some mice would presumably have survived. A minority gave very high counts and would presum-

ably have died, e.g. the mouse marked by an arrow (Fig. 10c) which was moribund when killed.

Another set of counts was done on mice given 0.05 LD₅₀ ($10^{5.7}$ organisms) of *Salm. paratyphi* B by intraperitoneal injection. As this dose was unlikely to cause any fatal infections, all the inoculated organisms must have been ineffective on the above hypothesis. *Salm. paratyphi* B was used, rather than *Salm. typhimurium*

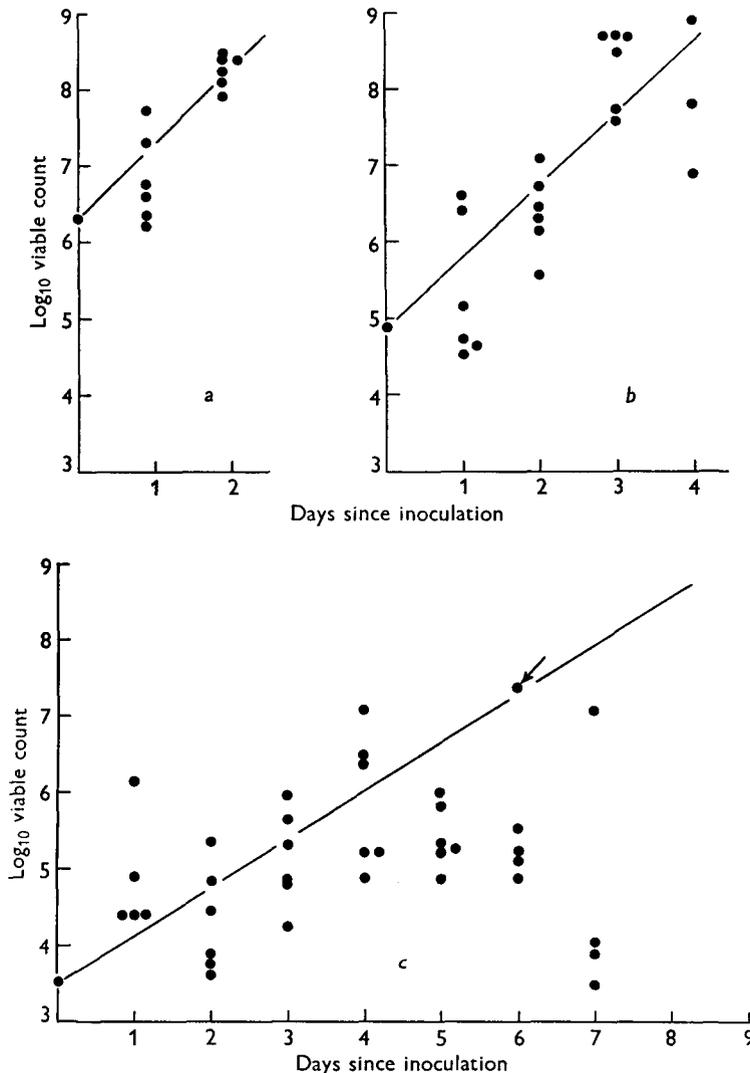


Fig. 10. Growth curves from Expt. 1 where three groups of mice were inoculated with $10^{6.3}$, $10^{4.9}$ and $10^{3.5}$ organisms, respectively. Each dot gives the viable count on the pooled blood, lungs, heart, liver, kidneys and spleen of one mouse. In Fig. 10c, a proportion of mice would presumably have survived since the rate of increase fell steadily from the third day after inoculation in most of the mice. The mouse marked by an arrow was moribund when killed. Each line joins the point on the ordinate corresponding to the inoculum with a point whose coordinates are $10^{8.75}$, the observed terminal viable count (Fig. 9), and the geometric mean death time observed in the main part of the experiment (Table 3).

because its LD_{50} was about 10^7 (Meynell & Stocker, 1957) and it was supposed that if ineffective organisms were inactivated or restrained, this would be more marked, the lower the virulence of the organism. Fig. 11 shows that the count, far from falling initially, rose for several days from a point slightly greater than that corresponding to the inoculum until each mouse contained about $1 LD_{50}$. The count then fell. Hence, viable counts could not have revealed the existence or manner of growth of an effective fraction, p , of inoculated organisms if this was present as predicted by the hypothesis of independent action, owing to the persistence and multiplication of ineffective organisms after inoculation.

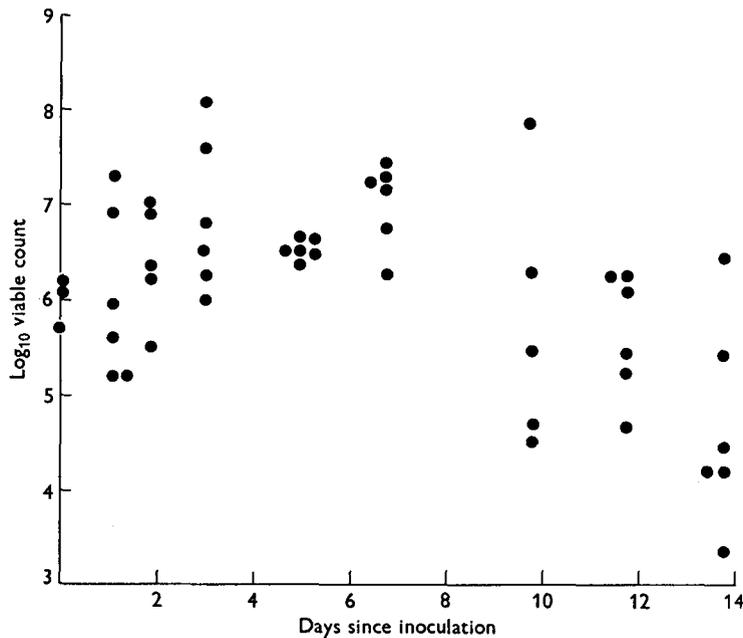


Fig. 11. Growth curve for mice inoculated with $0.05 LD_{50}$ ($= 10^{6.7}$ organisms) of *Salmonella paratyphi* B. This dose was unlikely to cause any fatal infections. The pair of points next to the ordinate are from counts made 1.5 hr. after inoculation.

DISCUSSION

The relationship between dose and death time for doses less than the LD_{50}

If inoculated organisms are completely independent, most hosts fatally infected by doses $\leq 1 LD_{50}$ should die after approximately the same mean length of time, since each will usually die following the multiplication of only one effective organism. This was observed in Expts. 1–3 (Figs. 3, 4), and these findings may be taken as further evidence in support of the hypothesis that inoculated organisms act independently.

Bryan has shown that some titrations of the Rous sarcoma virus (Bryan, Calnan & Moloney, 1955; Bryan, 1956) give apparently aberrant points for doses $\leq ED_{50}$; these are explicable on the above hypothesis. This system may belong to the limiting class, where the host is completely susceptible ($p = 1$) and the latent period tends

to constancy at doses $\leq 1 \text{ ED}_{50}$ because each host usually receives only one virus particle. However, as Epstein (1956) found by microscopy that each ED_{50} contained *ca.* 50 particles, the value of p may only be of the order of 0.015.

*The applicability of the proposed model to mice fatally infected
by Salmonella typhimurium*

Many infective systems yield a linear relation between mean latent period and log dose (Table 1) and the same relationship was observed in eight experiments with mice given *Salm. typhimurium* by intraperitoneal injection. The model given in the Introduction provides an explanation for this finding. It postulates that the organisms responsible for the production of the response increase *in vivo* at a constant rate and that the response will certainly occur when their number reaches a critical concentration.

The terminal viable count. The counts performed shortly after death on mice of differing resistance which had been challenged by doses of varying size, showed that the terminal viable count was approximately constant at $10^{8.75}$, regardless of the size of the dose and the resistance of the mice (Fig. 9). This finding strongly suggests that a critical concentration does exist and it will be shown below that the figure of $10^{8.75}$ agrees well with other observations. Berry, de Ropp, Fair & Schur (1956) reported a similar finding for normal and for vaccinated mice given various doses of *Salm. typhimurium* by intraperitoneal injection although their counts, done on whole carcasses disintegrated in a Waring blender, gave a mean figure of *ca.* $10^{7.6}$ organisms per mouse. Hobson (1957) also working with this system, obtained a figure of *ca.* $10^{8.5}$ from counts on the spleen, liver, kidneys, lungs and heart blood of mice dying 4–21 days after challenge by a dose of 10 organisms. These figures are markedly less than the toxic LD_{50} (*ca.* 10^{10} organisms) for heat-killed salmonella given by either intraperitoneal or subcutaneous injection (Felix, 1938 and our unpublished data).

Experiments with highly susceptible mice. Three experiments (nos. 4–6) were performed on mice that were almost completely susceptible ($\text{LD}_{50} = 3$ organisms; $p \sim 0.2$) so that the effective proportion of the inoculum was relatively very large. The model was almost certainly applicable to these experiments since published results show that increase *in vivo* is exponential (Schneider & Zinder, 1956; Hobson, 1956) and that the terminal viable count is constant and independent of death time (Hobson, 1957).

In our experiments, a lag of up to 1 day has to be postulated between the time C was reached and the occurrence of death. Expt. 4 shows this most clearly. A dose of 10^3 organisms increasing with an inferred doubling time of 3.2 hr. would reach the observed terminal count of $10^{8.75}$ in 3.9 days whereas the observed value of \bar{T} was 5 days.

Experiments with mice of intermediate resistance. In the remaining experiments (nos. 1–3, 7 and 8), the LD_{50} was ≥ 40 organisms and the mice were therefore at least moderately resistant. As mentioned in the Introduction, earlier results suggested that the length of the death times following inoculation of doses $\geq 1 \text{ ED}_{50}$ would be governed by the growth of a fraction of the inoculum far larger than that ($= p$)

predicted by the simple form of the hypothesis of independent action from the size of the ED_{50} . The following argument showed this to be so. Growth curves for the organisms whose multiplication determines the length of the latent period were constructed, first, by estimating their doubling time *in vivo* from the observed slope of the death time/log dose curve and, then, by comparing growth curves with the appropriate slope with those produced by the multiplication either of the fraction, p , or of any larger fraction of the inoculum. In Expts. 2 and 3, the slope of the death time/log dose curve (Fig. 4) gave a doubling time *in vivo* of 36.5 hr. The LD_{50}

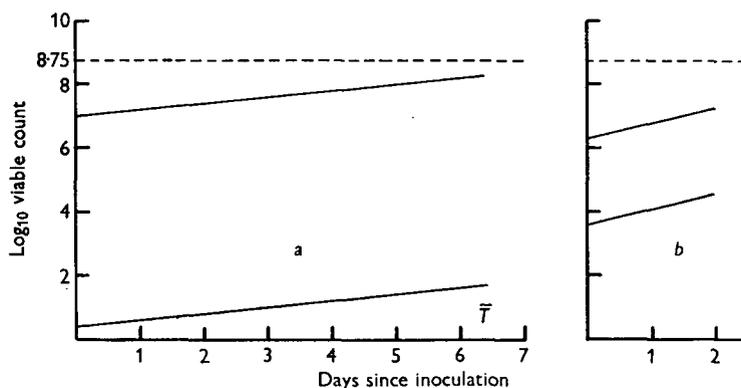


Fig. 12. Hypothetical growth curves for Expts. 2 and 3 (Fig. 12a) and Expt. 1 (Fig. 12b), derived from the inferred doubling time *in vivo*, on the assumption that the length of the death time is determined either by the growth of all the organisms inoculated (upper curves) or by the growth of only the fraction, p , predicted by the simple form of the hypothesis of independent action.

was 3.2×10^6 organisms and the value of p was therefore *ca.* 2.2×10^{-7} . Fig. 12a shows the growth curves with doubling time of 36.5 hr. (i) for the multiplication of all the organisms inoculated when a total of 10^7 is given, and (ii) for the multiplication of the postulated effective fraction = $10^7 \times 2.2 \times 10^{-7} = 10^{0.34}$ effective organisms. The figure shows that the first curve reaches a value of $10^{8.3}$ at 6.3 days after inoculation, the mean death time observed in the experiment. As the observed terminal viable count was $10^{8.75}$, the fit of this line is good. The curve for the effective fraction (lower curve in Fig. 12a) clearly does not reach the observed terminal concentration until long after the observed mean death time. The same calculations were made for Expt. 1 with the result shown in Fig. 12b where the count, assuming the whole inoculum to be effective, is $10^{7.2}$ at 2 days, the observed mean death time. In both figures the fit is better if it is supposed that the count increased two- to three-fold in the first 1.5 hr. after inoculation as it did in mice infected with *Salm. paratyphi* B (Fig. 11). Therefore, it appears that the length of the death time of a fatally infected partially resistant mouse is determined, not by the rate of growth of the postulated effective fraction, but by the growth of *all* the organisms inoculated. The growth curves for mice surviving challenge show that this could well occur owing to the growth of all the inoculated organisms (or their progeny) being 'triggered off' by the activity of the fraction, p . All organisms in

hosts surviving inoculation must, by definition, be ineffective: yet Figs. 10c and 11 show that for 2–3 days after inoculation their growth curves are indistinguishable from those postulated for effective organisms. Hence, a fall in resistance, produced by the fraction p , beginning a few days after inoculation, would act upon ineffective organisms which had increased almost exponentially since inoculation in the manner postulated by the model. The growth curves for fatally infected mice would then be expected to show the whole inoculum increasing at a constant rate from the time of inoculation. This is in fact observed (Figs. 10a, b).

Fig. 10 also shows that excellent agreement exists between independent measurements made on individual dose groups in Expt. 1; for the points of the growth curve for each group lie around a line joining a point on the ordinate corresponding to the whole inoculum with another point whose co-ordinates are: (i) the geometric mean death time for the group in question (obtained from Table 3) and (ii) $10^{8.75}$, the observed terminal viable count (Fig. 9). However, inconsistencies appeared when the data for all dose groups in Expt. 1 were compared; the estimated doubling time *in vivo* obtained from Fig. 3 (15.5 hr.) did not agree with the estimates obtained from the straight lines fitted to the growth curves in Fig. 10. These estimates are respectively 7.8, 7.8 and 11.4 hr. Conversely, the slope of the death time/log dose curve yielding a doubling time of 7.8 hr. was obviously flatter than that shown in Fig. 3 if the points for all doses $\geq 1 \text{ LD}_{50}$ are considered to fall around the same straight line, i.e. if, as has been tacitly assumed up to now, all fatal infections follow the same course, regardless of the size of the dose. This is known not to be true of some infections (such as infection of the mouse by *Mycobacterium tuberculosis*); and reinspection of Fig. 3 suggested that it may also not be true of our system since the points for doses $\geq 1 \text{ LD}_{50}$ could be considered to show two straight lines of differing slope which joined at a dose of $10^{4.8}$ organisms. This view was supported by the finding that the doubling time of 7.8 hr. obtained from the growth curves for mice given $10^{4.8}$ and $10^{6.3}$ organisms gave a slope for the death time/log dose curve which was a good fit to the four points for doses $\geq 10^{4.8}$ shown in Fig. 3.

One seemingly implausible assumption made by the model is that i , the rate of increase *in vivo*, remains constant. The viable counts on fatally infected mice show that this could be so but the validity of the assumption can be tested in another way. If the rate of increase fell in fatally infected mice at some time after challenge, its effect might be detected, possibly more accurately than by viable counts, by examining the distribution of death times for mice belonging to each dose group. In most reported systems, the distribution is log normal as found here (Fig. 2) so that a plot of probit cumulative mortality against log time since inoculation gives a straight line. However, the curves for groups given relatively small doses may be discontinuous as shown in the inset of Fig. 2 (Bryan & Beard, 1940; Cavalli & Magni, 1947; McKee, Rake, Donovan & Jambor, 1949; Beard, Sharp & Eckert, 1955: the latter discuss the treatment of these distributions). This implies that at a varying time after inoculation, which is related to the size of the dose, the hosts then surviving take longer to respond than would have been expected from the behaviour of those which had already responded. This effect can be reasonably

ascribed to a fairly sudden fall in the rate of increase *in vivo* which causes the response of the remaining hosts to be delayed to varying degrees according to the extent to which their infections had already progressed, so leading to a fall in the slope of the distribution. None of the data from our experiments show such discontinuities, suggesting either that the rate of increase did not change in fatally infected mice (the possibility postulated by the model and confirmed by the growth curves) or perhaps, that the rate fell uniformly in all members of each dose group before any died.

The direct relation between the size of the LD_{50} and the slope of the death time/log dose curve throws some light on the type of event determining the degree of resistance in this system. On the assumptions made above, this relation shows that the value of p , the mean probability per inoculated organism of multiplying sufficiently to initiate a response, is directly related to i , the rate of increase *in vivo* which is also the mean probability per organism of producing two viable progeny per unit time.* Hence, if p and i are governed by the same mechanism, the degree of resistance must be determined by *successive* random events occurring throughout the latent period. In the present system, one of these events is believed to be the formation of an abscess, usually in the liver, resulting from the multiplication of only one organism (Meynell & Stocker, 1957, Table 3). The probability per inoculated organism of forming a macroscopically visible abscess in an internal organ is often of the same order as the probability per inoculated organism of causing a fatal infection, since abscesses are found frequently only in mice surviving inoculation with 0.1–1 LD_{50} of either *Salmonella paratyphi* B (Meynell & Stocker, unpublished) or *Staphylococcus aureus* (Gorrill, personal communication).

Conversely, the association between p and i excludes theories analogous to the 'target' theory discussed in radiobiology, where the degree of resistance can be assumed to be decided by the probability of the occurrence of a *single* event shortly after inoculation, a model which yields the same dose-quantal response curve for subjects of identical resistance as the hypothesis of independent action (Meynell, 1957*b*). A possible example is death of most of the inoculum owing to sensitization by serum factors with lodgement of the surviving organisms, corresponding to the effective fraction, p , in protected sites in which increase to a lethal extent could occur at a rate which was independent of the degree of resistance. In our system, rapid inactivation of the postulated ineffective organisms followed, in the case of fatally infected mice, by the survival and outgrowth of an effective fraction, is also excluded by the growth curves (Figs. 10, 11).

Finally, it is interesting to observe the differing extents to which a terminal breakdown in resistance disturbs the various tests of the simple form of the hypothesis of independent action. The predictions for the present experiments using

* This conclusion does not necessarily follow if two groups of hosts (e.g. normal and vaccinated) are inoculated with only *one* size of dose such that all respond, and are then found to have significantly different latent periods. For example, this result would also be obtained if i was the same for both groups while p differed. The latent period/log dose curves would then have the same slope but would be placed at different points on the dose axis. This relation has not yet been reported.

doses greater than 1 LD₅₀ are completely upset as the death time is seen to be governed, not by the postulated effective fraction, but by the whole inoculum. The experiments using mixtures of distinguishable variants (Meynell & Stocker, 1957) were less disturbed, since the results were in general agreement with prediction. Arguments based on the shape of the dose/quantal response curve (Meynell, 1957*b*) were not affected at all. The reason for these differences is that the first two experiments are greatly affected by the *nature* of the action exerted by the postulated effective fraction and by the fates of the ineffective fraction; whereas the shape of the dose/quantal response curve is not affected by the nature of the action, but solely by whether or not it occurs at random as postulated by the hypothesis.

SUMMARY

The growth of *Salmonella typhimurium* in mice was studied by performing viable counts on pooled viscera and, in the case of fatally infected mice, by determining the relation between size of dose and mean death time. The results suggested that this system conformed to a general model which postulates (*a*) that the organisms causing the response (death in the present experiments) increase *in vivo* at a rate which is constant for all doses, and (*b*) that the response is certain to occur when their number reaches a constant figure. It follows that logarithm of dose (for doses ≥ 1 ED₅₀) should be linearly related to mean latent period (the interval between inoculation and response). A search of the literature showed that this relation was observed in many reported infective systems.

The interpretation of the observations on partially resistant mice (LD₅₀ = 10^{2.5}–10⁶ organisms) was not straightforward for the hypothesis of independent action (Meynell & Stocker, 1957) states that only a random fraction of the inoculum usually initiates each response. Multiplication of this fraction is considered to cause a reduction in host resistance so that other organisms surviving *in vivo* began to increase in numbers and to contribute to the fatal infection. The observations showed that this could well occur as (i) the counts on all mice, whether fatally infected or not, increased exponentially for 2–3 days after inoculation; (ii) the growth curves of fatally infected mice showed exponential increase of the whole inoculum as postulated; and (iii) the length of the death time was found to be determined by the growth of the whole inoculum and not by the predicted random fraction. The viable count per fatally infected mouse at the time of death was approximately constant at 10^{8.75} organisms. There was no evidence that the course of the infection was influenced by organisms which may have been killed *in vivo* as the requirements of the model were satisfied by the observed behaviour of the viable organisms alone. The rate of increase of organisms in a fatally infected animal was found to be less, the greater the LD₅₀ of the host; which suggested that the degree of host resistance was determined by successive random events occurring after inoculation.

The latent period/log dose curve provides a new test of the hypothesis of independent action, since this predicts that most responses to doses equal to or less than 1 ED₅₀ are each initiated by only one inoculated organism. Hence, the latent

period should become approximately constant at such doses. This was found to be so in the present experiments.

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